

Hepatitis B virus X protein modulates peroxisome proliferator-activated receptor γ through protein–protein interaction

Youn-Hee Choi^a, Ha-il Kim^b, Je Kyung Seong^c, Dae-Yeul Yu^d, Hyeseong Cho^e,
Mi-Ock Lee^f, Jae Myun Lee^a, Yong-ho Ahn^b, Se Jong Kim^a, Jeon Han Park^{a,*}

^aDepartment of Microbiology and Brain Korea 21 Project of Medical Sciences, Institute for Immunology and Immunological Diseases, Seoul, South Korea

^bDepartment of Biochemistry and Molecular Biology, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, South Korea

^cLaboratory of Developmental Biology and Genomics, College of Veterinary Medicine, Seoul National University, Seoul 150-742, South Korea

^dKorea Research Institute of Bioscience and Biotechnology, Taejeon 305-600, South Korea

^eDepartment of Biochemistry, Ajou University School of Medicine, Suwon 442-749, South Korea

^fDepartment of Bioscience and Biotechnology, Sejong University, Seoul 140-747, South Korea

Received 8 August 2003; revised 19 November 2003; accepted 23 November 2003

First published online 24 December 2003

Edited by Felix Wieland

Abstract Ligand activation of peroxisome proliferator-activated receptor γ (PPAR γ) has been reported to induce growth inhibition and apoptosis in various cancers including hepatocellular carcinoma (HCC). However, the effect of hepatitis B virus X protein (HBx) on PPAR γ activation has not been characterized in hepatitis B virus (HBV)-associated HCC. Herein, we demonstrated that HBx counteracted growth inhibition caused by PPAR γ ligand in HBx-associated HCC cells. We found that HBx bound to DNA binding domain of PPAR γ and HBx/PPAR γ interaction blocked nuclear localization and binding to recognition site of PPAR γ . HBx significantly suppressed a PPAR γ -mediated transactivation. These results suggest that HBx modulates PPAR γ function through protein–protein interaction. © 2003 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Key words: Hepatitis B virus X protein; Peroxisome proliferator-activated receptor γ ; Transactivation; Protein–protein interaction

1. Introduction

Peroxisome proliferator-activated receptor γ (PPAR γ) belongs to the steroid hormone receptor superfamily and regulates cell growth, differentiation, insulin sensitization, and glucose homeostasis [1]. Upon receptor ligation, PPAR γ forms heterodimers with the retinoid X receptor (RXR) [2], which bind to specific DNA regions, called peroxisome proliferator response elements (PPRE), in the 5' flanking region of target

genes [3], and form complexes with coactivators and corepressors involved in PPAR/RXR-mediated transactivation [1].

Recently, it has been reported that the ligand-induced activation of PPAR γ shows anti-tumor effect by inducing growth inhibition, terminal differentiation, and apoptosis in various human cancer cells, including colon, prostate and breast cancer, and liposarcoma [4–8]. It was also reported that the ligand activation of PPAR γ induced growth inhibition and apoptosis in hepatocellular carcinoma (HCC) cells [9–11], however, its effect on virus-associated HCC has not been clarified.

Hepatitis B virus (HBV) is considered to be the major cause of hepatitis, cirrhosis, and HCC. Among the HBV proteins, hepatitis B virus X protein (HBx) is considered to contribute significantly to the mediation of pathological events and to the dysregulation of gene expression through protein–protein interactions with endogenous cellular proteins and transcriptional factors. The transactivatory role of HBx is thought to be mediated by its interaction with transcription factors, such as RPB5 of RNA polymerase [12], TATA binding protein [13], transcription factor IIB [14], bZIP protein [15], and RXR [16]. It has been also reported that HBx interferes with p53 by direct binding and by sequestering p53 in the cytoplasm, leading to the abrogation of p53-mediated cellular processes [17–19]. Wang et al. [20] reported that HBx inhibits sequence-specific DNA binding and thus blocks the transcriptional activity of p53. HBx is known to promote cellular transformation, as demonstrated by the immortalization of primary mouse hepatocytes [21] and by the development of hepatoma in HBx transgenic (HBx-TG) mice [22–24]. HBx is involved in the activation of signal transduction pathways, such as Ras-Raf-MAP kinase, PI3 kinase, protein kinase C, Src kinase, nuclear factor (NF)- κ B, and Jak1-STAT signal pathways, which lead to the activation and regulation of various genes that contribute to cellular proliferation and carcinogenesis [25].

In this study, we examined the effect of HBx on the growth inhibition induced by PPAR γ ligand in HCC cells. We also described that HBx bound to PPAR γ and HBx/PPAR γ interaction in the cytoplasm, and that this interfered with the nuclear localization and DNA binding of PPAR γ , leading to the

*Corresponding author. Fax: (82)-2-392 7088.

E-mail address: jhpark5277@yumc.yonsei.ac.kr (J.H. Park).

Abbreviations: PPAR γ , peroxisome proliferator-activated receptor γ ; HCC, hepatocellular carcinoma; HBx, hepatitis B virus X protein; HBV, hepatitis B virus; RXR, retinoid X receptor; PPRE, peroxisome proliferator response element; HBx-TG, hepatitis B virus X protein transgenic; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; NE, nuclear extract; DBD, DNA binding domain; LBD, ligand binding domain; NLS, nuclear localization signal; IFA, immunofluorescence assay; rHBx, recombinant hepatitis B virus X protein

suppression of the transcriptional activity of PPAR γ . Thus, our data show a novel association between HBx and PPAR γ , which may provide another important mechanism in the pathogenesis of HBV-associated HCC.

2. Materials and methods

2.1. Cell lines and culture

Chang liver (ATCC CCL-13), HepG2 (ATCC HB-8065) and HEK293 (ATCC CRL-1573) were obtained from the American Type Tissue Culture Collection (Manassas, VA, USA). Chang, HEK293, HepG2 and HepG2.2.15 cells [26] were maintained in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS). Chang X-34 cells are Chang liver cells expressing HBx under the control of tetracycline inducible promoter [19]. HBx was induced with 2 μ g/ml doxycycline (Sigma, St. Louis, MO, USA) in Chang X-34 cells.

2.2. Plasmids and plasmid construction

A series of green fluorescent protein (GFP)–PPAR γ fusion constructs (pEGFP-PPAR γ F, D, DL, L) shown in Fig. 2B were generated by polymerase chain reaction (PCR) amplification of the PPAR γ gene with specific primer sets, corresponding to the PPAR γ full length (F; residues 1–475), PPAR γ DNA binding domain (DBD) (D; residues 94–181), PPAR γ DNA and ligand binding domain (LBD) (DL; residues 94–475), and PPAR γ LBD (L; residues 281–475). These were then subcloned into the *SacI*–*Sall* site of pEGFP vector (Clontech, Palo Alto, CA, USA). To generate His fusion recombinant HBx (rHBx) protein, an amplified HBx fragment was cloned into *EcoRI*–*HindIII* sites of pRSET A (Invitrogen, Carlsbad, CA, USA). PPRE $_3$ –thymidine kinase (tk)–LUC and pCMX-PPAR γ [27] were kindly provided by Dr. R. Evans. The HBx DNA construct, pSVX, and its frameshift mutant, pSVX κ B [28], were used for the transfection experiments. To express HBx with a hemagglutinin (HA) epitope tag at the N-terminal (pCMV-HA–HBx), the HBx fragment was amplified by PCR and subcloned into the *EcoRI*–*KpnI* site of pCMV-HA plasmid (Invitrogen).

2.3. Generation and infection of recombinant adenoviruses

The construction of adenoviral vectors was performed as described previously [29]. The adenoviral plasmids (pAdEasy-1, -2) and the shuttle vectors (pShuttle, pShuttle-CMV, pAdTrack, and pAdTrack-CMV) were constructed by subcloning the PCR product of HBx. Approximately 3×10^6 HEK293 cells were plated in 25 cm 2 flasks 24 h before transfection, by which time they had reached 50–70% confluency. 4 μ g of recombinant adenoviral vector DNA, digested with *PacI*, was then transfected using 20 μ l LipofectAMINE (Gibco BRL). The transfected cells were monitored for GFP expression and harvested 7 days after transfection. Cells were collected and lysed by three cycles of freezing/thawing. A concentrated adenovirus fraction was prepared by CsCl gradient centrifugation and stored with 2 \times storage buffer (10 mM Tris, pH 8.0, 100 mM NaCl, and 0.1% bovine serum albumin (BSA), and 50% glycerol) at –20°C. 1 μ l of virus stock was used to infect $3\text{--}5 \times 10^6$ cells. The efficiencies of the infections were monitored by GFP expression.

2.4. rHBx protein expression

His fusion rHBx protein was expressed in *Escherichia coli* strain BL21(DE3) and purified on nickel agarose bead. Protein expression, preparation of inclusion body protein, and refolding of denatured protein were performed as described previously [30].

2.5. HBx-TG mice

HBx-TG mice were generated [24] and maintained as described previously [31]. We used HBx homozygote transgenic mice and age-matched wild-type controls. The protocol for mice maintenance was approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University, according to the Guide for Animal Experiments edited by the Korean Academy for Medical Association.

2.6. Antibodies and reagents

Antibodies against PPAR γ (sc-7273), HA (sc-805, sc-7392), and

GFP (sc-9996, sc-8334) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antiserum against HBx was prepared by intraperitoneal immunization to BALB/c mice with rHBx emulsified with complete and sequentially incomplete Freund's adjuvant and its reactivity was screened by Western blot and enzyme-linked immunosorbent assay (ELISA). The antibodies against α -tubulin and histone were from Oncogene (Darmstadt, Germany) and Chemicon International Inc. (Temecula, CA, USA), respectively. Protein G/A was purchased from Gibco, and protein assay reagent and enhanced chemiluminescence reagent were from Pierce (Rockford, IL, USA). Troglitazone, provided by SanKyo (Tokyo, Japan), was prepared as previously described [32].

2.7. Cell proliferation assay

Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [33]. Briefly, 1×10^3 cells were seeded into 96-well plates and treated with troglitazone and incubated for different time periods. After treatment, 50 μ l MTT solution (1 mg/ml in phosphate-buffered saline (PBS)) was added to each well, and incubated for 4 h at 37°C. The plate was then centrifuged at 3000 rpm for 10 min, the supernatant was discarded, and the remaining adhered cells were dissolved in 50 μ l dimethyl sulfoxide (DMSO) (Sigma). The optical density (OD) of each well was measured at 570 nm using an ELISA reader.

2.8. Reverse transcriptase (RT)-PCR

Total RNA was prepared using RNeasy Kit (Qiagen Inc, Chatsworth, CA, USA). Reverse transcription for cDNA was performed from 5 μ g total RNA using 2 μ g random hexamer (Amersham Pharmacia Biotech Inc., Uppsala, Sweden), 1.25 mM deoxyribonucleoside triphosphate (dNTP) (Boehringer-Mannheim, Mannheim, Germany) and with or without addition of 200 U M-MLV RT (Gibco BRL). PCR was performed using 0.25 mM dNTP, 0.25 U Taq polymerase (Perkin Elmer, Norwalk, CT, USA), 10 pmol primer pair and 3 μ l cDNA with a thermal cycler. The primer sequences were as follows: HBx, 5'-CGGAATTCGGATGGCTGCTAGGAT-3' (sense) and 5'-CGGGGTACCCCGT-TAGGCAGAGGT-3' (antisense); β -actin, 5'-TTGTAACCAACTGGGACGATTGG-3' (sense) and 5'-GATCTTGATCTTCATGG-TGCTAGG-3' (antisense). PCR cycling conditions were as follows: denaturation at 92°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min.

2.9. Immunoprecipitation and Western blot analysis

For the HBx and PPAR γ co-immunoprecipitation assay, Chang X-34 cells were treated with 2 μ g/ml doxycycline, and then lysed with radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris–Cl, pH 7.4, 100 mM NaCl, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 0.2% NP-40 and 2 mM MgCl $_2$ supplemented with protease inhibitor cocktail (Sigma). To detect the intracellular interactions of HBx with the deletion mutants of PPAR γ , HEK293 cells were cotransfected with pCMV-HA–HBx and pEGFP-PPAR γ F, D, DL, or L, respectively. Cell lysates (500 μ g) were then incubated with 1 μ g anti-HA monoclonal antibody and incubated with protein G/A, and Western blotting was performed using polyclonal anti-HA, anti-PPAR γ , and anti-GFP antibodies. To detect the HBx and PPAR γ protein expression in the mouse liver tissues, tissues were fractionated into cytosolic and nuclear extracts [32] and analyzed by quantitative Western blot analysis using HBx antiserum and anti-PPAR γ antibody.

2.10. Immunofluorescence assay (IFA)

IFA was used to investigate subcellular localization of the HBx and PPAR γ . Briefly, Chang liver cells were seeded into Lab-Tek two-chamber glass slides (Nalge Nunc, Naperville, IL, USA) and transiently transfected with pEGFP-PPAR γ and pCMV-HA–HBx. Cells were incubated with 20 μ M troglitazone for 24 h, washed and fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% saponin (Sigma) in PBS. Cells were then incubated with anti-HA antibody and then with Cy3-conjugated IgG (Zymed Laboratories Inc., San Francisco, CA, USA). After several washes, cells were mounted using mounting medium containing 4,6-diamino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and visualized under a confocal microscope, LSM 510 (Carl Zeiss, Jena, Germany), or fluorescence microscope (Olympus America Inc., Melville, NY, USA) with MetaMorph software (Universal Imaging Corporation, Downingtown, PA, USA).

2.11. Electrophoretic mobility shift assay (EMSA)

Preparations of nuclear extract (NE) and EMSA were carried out as previously described [32]. For the binding reaction, 5 μ g NE was incubated with the 32 P-labeled probe in binding buffer containing 10 mM Tris, pH 7.5, 100 mM KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml BSA, and 5% glycerol at room temperature for 30 min. For the competition experiments, a 100 molar excess cold probe was added to the reaction mixture. To examine the effect of HBx on the DNA binding activity of PPAR γ , rHBx was added to the reaction mixture for 30 min, and then the probes were added. For SP-1 binding, 2 μ g

NE was incubated with the labeled probe. The sequence of the oligonucleotides used as probes was as follows: CYP4A-PPRE, 5'-TG-AACTAGGGTAAAGTTCA-3'; SP-1, 5'-GATCGATCGGGC-GGGGCGAG-3'.

2.12. Luciferase reporter assay

Chang liver cells (2×10^5) were seeded onto 12-well culture plates and transfection was carried out using LipofectAMINE (Gibco BRL), according to the manufacturer's instruction. The total amount of DNA was adjusted to 1 μ g; 500 ng PPRe₃-tk-LUC, 200 ng pCMX-PPAR γ , 200 ng pSVX or pSVX κ B, 100 ng pCMV- β -gal,

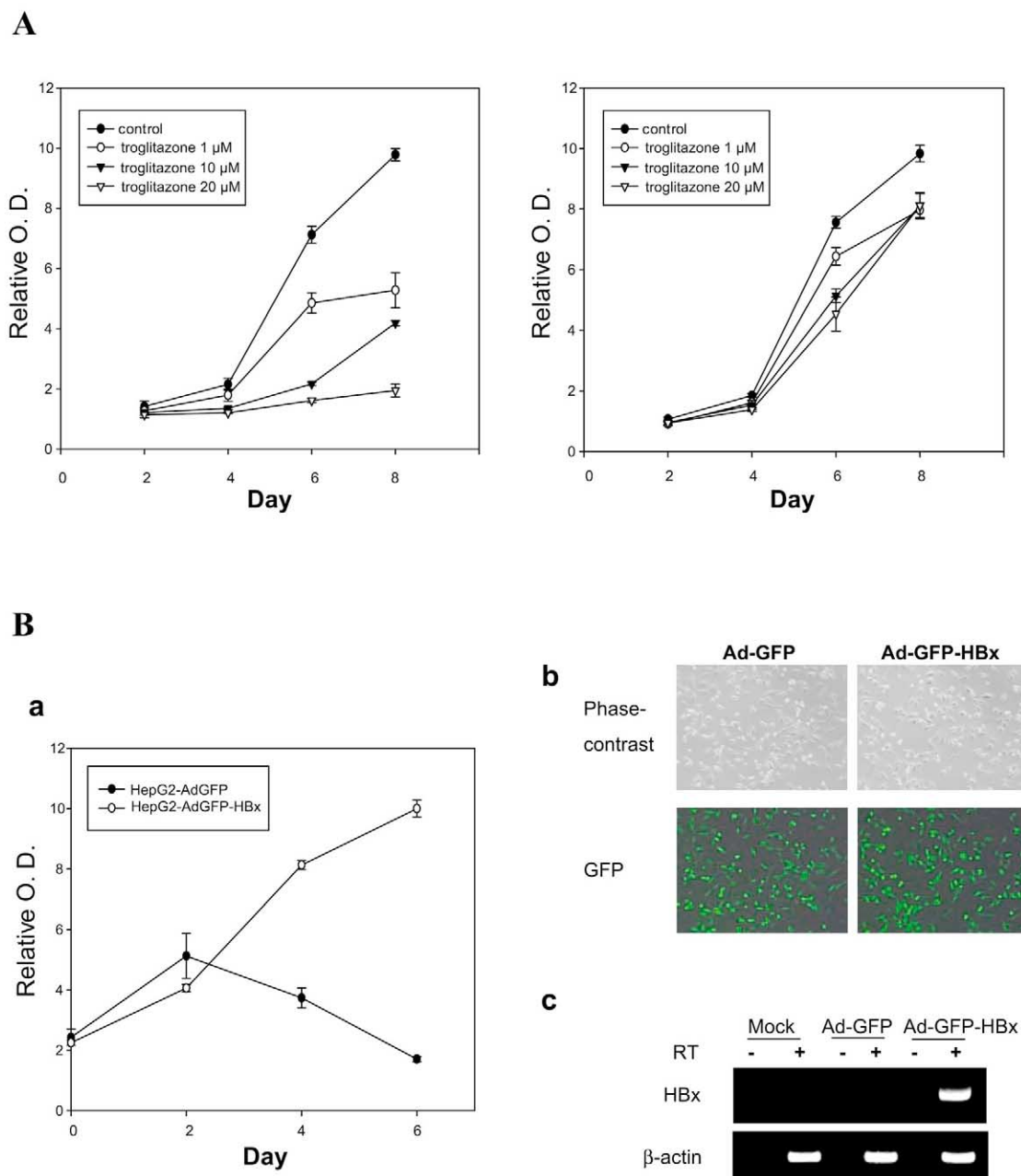


Fig. 1. HBx abolishes the inhibitory effect of troglitazone on cellular proliferation. A: HepG2 (left panel) and HepG2.2.15 (right panel) cells were treated with 1.0–20 μ M troglitazone for the indicated times, and cell proliferation was determined by MTT assays. B: (a) HepG2 cells transduced with recombinant adenovirus carrying HBx (Ad-GFP-HBx) or control virus (Ad-GFP) were treated with 20 μ M troglitazone for the indicated times and cell proliferation was determined by MTT assays. Results are represented as relative OD compared with the control and normalized values are shown as means \pm S.D. Assays were performed at least three times using quadruplicate samples, and the data of a representative experiment are shown. (b) Transduction efficiency of Ad-GFP and Ad-GFP-HBx into HepG2 cells. GFP microscopic finding showed that more than 95% HepG2 cells were transduced in all the fields observed. (c) HBx expression after transduction of Ad-GFP-HBx. After transduction of Ad-GFP and Ad-GFP-HBx, total RNA was extracted from transduced HepG2 cells and treated with RNase-free DNase and then analyzed by RT-PCR with (+) or without (–) RT.

and empty vectors. After 3 h of transfection, the cells were washed and incubated in a complete medium containing 20 μ M troglitazone for 24 h. Cells were then washed and lysed using 1 \times lysis buffer (Promega, Madison, WI, USA). Lysates were measured for luciferase activity using luciferin (Promega), and β -galactosidase activity was measured as an internal control.

3. Results

3.1. HBx suppresses PPAR γ -induced growth inhibition in HCC cells

HepG2 cells express PPAR γ , and their growth is inhibited by troglitazone [10,11]. However, in this study, we found that HepG2.2.15 cells, which had been established by transfecting four tandem copies of the HBV genome into HepG2 cells [26], were resistant to troglitazone. When cells were treated with troglitazone for different times, the proliferation of HepG2 cells significantly decreased in a dose-dependent manner. However, HepG2.2.15 cells appeared to be more resistant to troglitazone-induced growth inhibition than HepG2 cells (Fig. 1A). To exclude the possibility that the difference of sensitivity to troglitazone was due to different sublines used, we examined HepG2 infected with recombinant adenovirus carrying HBx (HepG2-Ad-HBx). As shown in Fig. 1B, the growth rate of HepG2-Ad-HBx cells was significantly higher than that of HepG2 cells infected with Ad-GFP empty vector. Thus, these results demonstrate that HBx significantly suppressed troglitazone-induced growth inhibition in HCC cells.

3.2. HBx binds to PPAR γ in vivo

It was reported that HBx mediates its biological activity through protein–protein interactions with transcription factors [12–16]. To determine whether HBx interacts with PPAR γ , an immunoprecipitation assay was performed. Chang X-34 cells, which express HBx-HA under the control of tetracycline inducible promoter, were incubated with doxycycline for various times. Cell lysates were immunoprecipitated with an anti-HA monoclonal antibody, and then analyzed using an anti-PPAR γ antibody. The HBx–PPAR γ complex was found to increase in proportion to the amount of HBx expressed by doxycycline, in a time-dependent manner, although the endogenous PPAR γ protein level was unaffected by HBx (Fig. 2A). To determine which domain of PPAR γ is involved in this binding, HEK293 cells were transfected with pCMV-HA–HBx and a series of GFP–PPAR γ fusion constructs (Fig. 2B). Cell lysates were then immunoprecipitated with an anti-HA antibody, and analyzed with an anti-GFP antibody. As shown in Fig. 2C, upper panel, PPAR γ F, D and DL were found to be associated with HBx. This finding demonstrates that HBx binds to PPAR γ , and that the DBD–hinge region of PPAR γ is critical for HBx binding, because GFP–PPAR γ D is composed of DBD and hinge region.

3.3. HBx interferes with the nuclear localization of PPAR γ

DBD of PPAR γ is involved in the binding to PPRE, and the hinge region participates in nuclear localization and the formation of heterodimers [34,35]. Having observed that HBx binds to the DBD–hinge region of PPAR γ , we investigated whether the HBx–PPAR γ interaction affects the nuclear localization of PPAR γ . Chang liver cells were cotransfected with pEGFP–PPAR γ and pCMV-HA–HBx, and IFA was then performed. In the absence of HBx, PPAR γ was found to be localized mainly in the nucleus, regardless of troglitazone

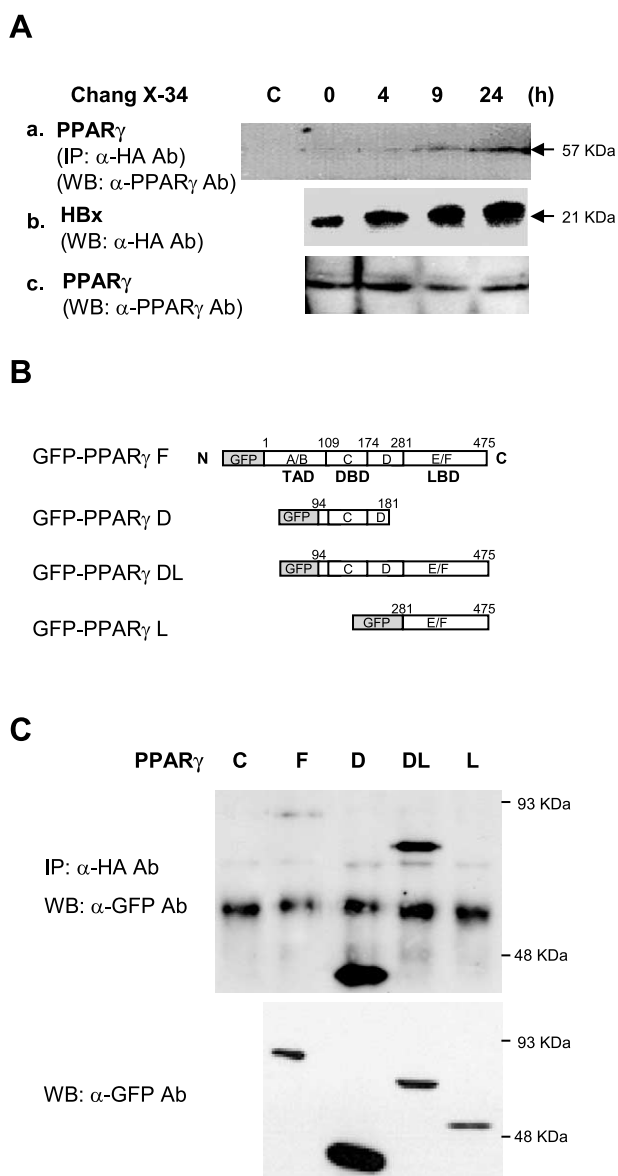


Fig. 2. Interaction of PPAR γ with HBx in vivo. A: After treating Chang X-34 cells with 2 μ g/ml doxycycline, (a) the cell lysate was immunoprecipitated with anti-HA monoclonal antibody and analyzed on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Western blot was performed with anti-PPAR γ antibody. C, immunoprecipitation with normal mouse immunoglobulin. (b) Western blot with anti-HA antibody. (c) Western blot with anti-PPAR γ , showing that an equal amount of PPAR γ had been loaded. B: A schematic diagram of four GFP–PPAR γ fusion constructs encoding the indicated domain. C: After transfection with the HA–HBx and GFP–PPAR γ fusion constructs (PPAR γ F, D, DL, or L), HEK293 cells were lysed and the cell lysates were immunoprecipitated with mouse anti-HA monoclonal antibody. Western blot was performed with rabbit anti-GFP polyclonal antibody after immunoprecipitation (upper panel). C, immunoprecipitation with normal mouse immunoglobulin. The same amount of cell lysates was analyzed by Western blot with mouse anti-GFP monoclonal antibody (lower panel).

treatment (Fig. 3A, upper panel). However, in the presence of HBx the localization of PPAR γ changed from the nucleus to the cytoplasm, although some PPAR γ remained in the nucleus (Fig. 3A, lower panel). As shown in Fig. 3B, this cytoplasmic PPAR γ was found to be colocalized with HBx (merged; yellow color).

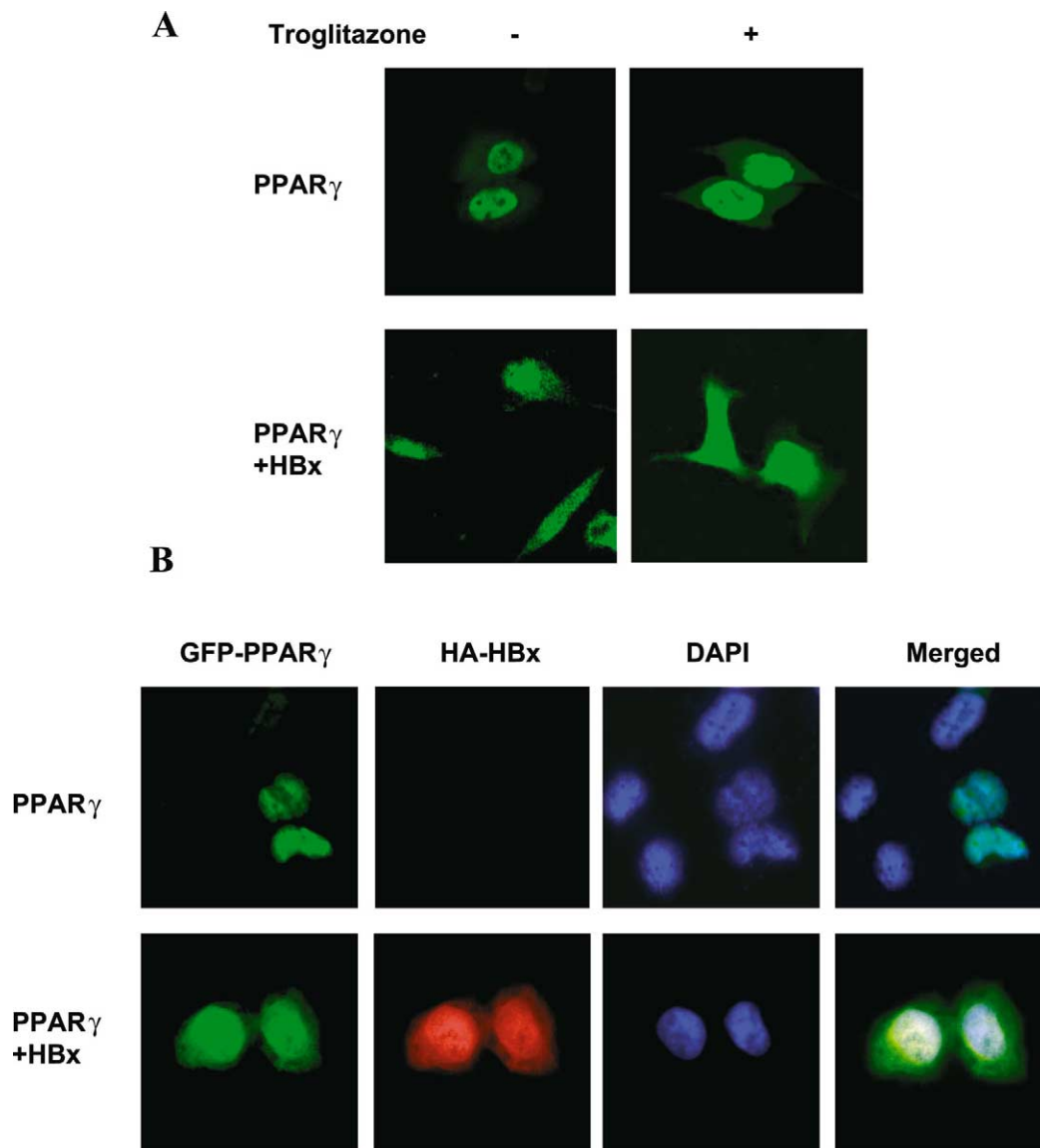


Fig. 3. Colocalization of PPAR γ with HBx. A: pEGFP-PPAR γ with or without pSVX-HBx was transfected into Chang liver cells and treated with or without 20 μ M troglitazone for 20 h, cells were observed under a confocal microscope. The partial cytoplasmic sequestration of PPAR γ was observed in the presence of HBx. Upper panel, pEGFP-PPAR γ transfected Chang liver cells; lower panel, pEGFP-PPAR γ and pSVX-HBx cotransfected Chang liver cells. B: pEGFP-PPAR γ was transfected into Chang liver cells with or without pCMV-HA-HBx. After incubation with 20 μ M troglitazone for 20 h, the cells were stained and analyzed under a confocal microscope. Cells were visualized with triple colors, i.e. GFP (PPAR γ), and rabbit anti-HA antibody and Cy3-conjugated anti-rabbit IgG (HBx), and DAPI (nucleus), and merged.

3.4. PPAR γ expression is reduced in the nucleus of HBx-TG mouse liver

To determine whether this interference with the nuclear localization of PPAR γ by HBx occurs *in vivo*, we prepared the mouse liver tissue extracts from HBx-TG and age-matched control C57BL/6 which were fractionated into cytosolic, and nuclear extracts for the quantification, and then Western blot was performed using anti-PPAR γ antibody and HBx antiserum. Both proteins were detected in total and cytosolic compartments of HBx-TG. No significant difference was found in the PPAR γ expression in the total and cytosolic compartments between the HBx-TG and control C57BL/6 mouse liver tissues, however the expression of PPAR γ in the nuclear extracts of the HBx-TG was significantly lower than in that of the control (Fig. 4).

3.5. The DNA binding of PPAR γ is reduced in HBx-TG mouse liver

To determine whether reduced PPAR γ expression in the nucleus of HBx-TG mouse liver is associated with the DNA binding activity of PPAR γ , EMSA was performed with PPRE. NE was prepared from the livers of 13-month-old HBx-TG mice and age-matched C57BL/6 mice. As shown in Fig. 5A, the NE from the control C57BL/6 mouse livers showed significant DNA binding to PPRE, however the DNA binding of PPAR γ was completely abrogated in the NE of HBx-TG mouse livers. Since HBx interacts with the PPAR γ DBD–hinge region, we also investigated whether HBx inhibits the DNA binding activity of PPAR γ by performing EMSA using NE from control C57BL/6 mouse liver, which was preincubated with rHBx protein. Fig. 5B shows that

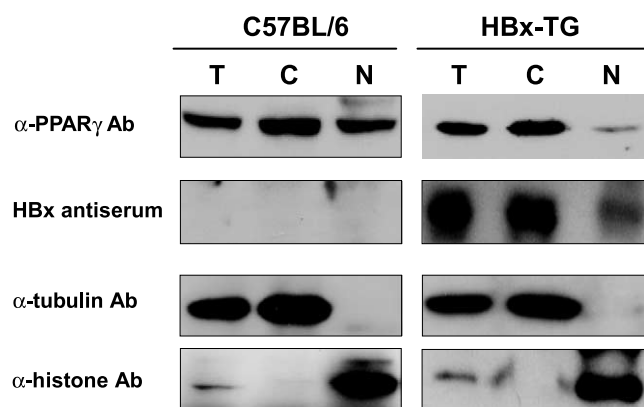


Fig. 4. Reduced PPAR γ protein level in the nucleus of HBx-TG mouse liver. Total (T: 100 μ g), cytosol (C: 100 μ g) and nuclear (N: 25 μ g) extracts were analyzed using anti-PPAR γ antibody and anti-serum against HBx. Western blotting with anti- α -tubulin and anti-histone antibodies were used to confirm the fractionation.

rHBx significantly reduced the DNA binding activity of PPAR γ . Overall, it appears that binding of HBx to PPAR γ interferes with the nuclear localization and the DNA binding activity of PPAR γ .

3.6. HBx suppresses the transcriptional activity of PPAR γ

To determine whether the interruption of PPAR γ localization by HBx influences the transcriptional activity of PPAR γ , a luciferase reporter assay was performed. Compared with Chang liver cells transfected with PPRE₃-tk-LUC alone, luciferase activity was found to be increased by PPAR γ over-expression, and this activity was enhanced by troglitazone. However, luciferase activity was markedly reduced by HBx even in the presence of troglitazone, though this suppressive effect was not observed by the HBx mutant (Fig. 6A). In addition, this suppressive effect of HBx on transcriptional activity was dose-dependent (Fig. 6B). These results suggest that HBx inhibits the transcriptional activity of PPAR γ .

4. Discussion

PPAR γ is a nuclear hormone receptor with diverse functions, which are critical for maintaining cellular processes [36]. Several studies have demonstrated that the ligands of PPAR γ may induce apoptosis and growth inhibition in HCC cells, which suggests that PPAR γ has an anti-cancer effect [9–11]. In the present study, the proliferation of troglitazone-treated HepG2 cells decreased significantly in a dose-dependent manner, which agrees with previous reports [10,11]. Interestingly, HepG2.2.15 and HepG2 cells infected with Ad-GFP-HBx were resistant to troglitazone, suggesting that the introduction of the HBV genome or HBx appeared to convert troglitazone-susceptible cells to troglitazone-resistant cells.

PPAR γ is composed of two zinc finger motifs, nuclear localization signals (NLS), a ligand binding pocket, and several activation functions (AF) [35]. DBD has two zinc finger motifs that can bind directly to DNA, and the hinge region contains NLS. Our immunoprecipitation assay with Chang X-34 cells demonstrated that the protein–protein interaction occurred between HBx and the PPAR γ , and this interaction occurred through DBD–hinge region of PPAR γ . Since the DBD–hinge region participates in the interaction with HBx,

this interaction might modulate the nuclear localization and DNA binding of PPAR γ . DBD is the most highly conserved region between other members of steroid hormone receptor superfamily. This fact leads to the possibility that HBx may interact with and affect other PPAR γ isoforms or other steroid receptor members. Previously, it was reported that the DBD of several steroid hormone receptors could interact with DBD-associating proteins. Thioredoxin, one of the DBD-associating proteins, interacts with the DBD of glucocorticoid receptor [37], and the small nuclear ring finger protein SNURF directly binds to the DBD of androgen receptor [38]. In addition to these cellular proteins, viral proteins can also modulate steroid hormone receptors by direct protein–protein interaction. Hepatitis C virus core protein interacts with the DBD of RXR α [39] and HBx binds to the LBD of RXR [16]. Since PPAR γ forms a complex by heterodimerizing with RXR and binding to other coactivators or corepressors, it is also possible that PPAR γ indirectly binds to HBx through complex formation with other components. However, the strong dimerization of PPAR γ with RXR α is mainly mediated by the LBD of PPAR γ [40], and our data show an interaction between PPAR γ and HBx via the DBD–hinge region of PPAR γ , thus it is probable that HBx interacts with PPAR γ by direct protein–protein interaction. Moreover, our native gel shift assay of the in vitro binding of HBx to PPAR γ demonstrated a direct protein–protein interaction. To exclude the possibility that HBx binds to PPAR γ indirectly via interaction with RXR, we performed an in vitro binding assay with rHBx and three His-PPAR γ proteins corresponding to PPAR γ D, DL, and L. A native gel shift assay showed shifted bands in mixtures of HBx with PPAR γ D, or PPAR γ DL, but not in mixture of HBx with PPAR γ L (our unpublished observation). These results indicate that HBx binds directly to the DBD–hinge region of PPAR γ , which plays important roles

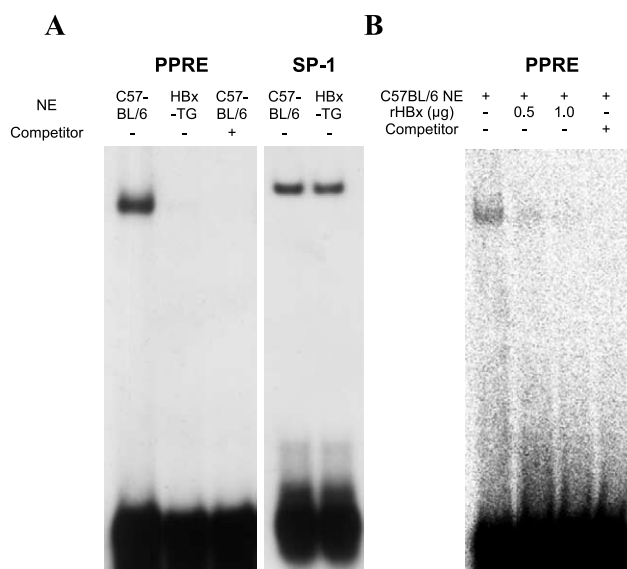


Fig. 5. The lower PPRE binding of NE from HBx-TG mouse liver tissues. A: NE (5 μ g) from control C57BL/6 and from HBx-TG mouse liver were analyzed by EMSA. For the competition assay, a 100 molar excess of the cold probe was added to the reaction mixture. SP-1 binding was used as control. B: NE from control C57BL/6 mouse liver was preincubated with rHBx at room temperature for 30 min before the addition of the probe.

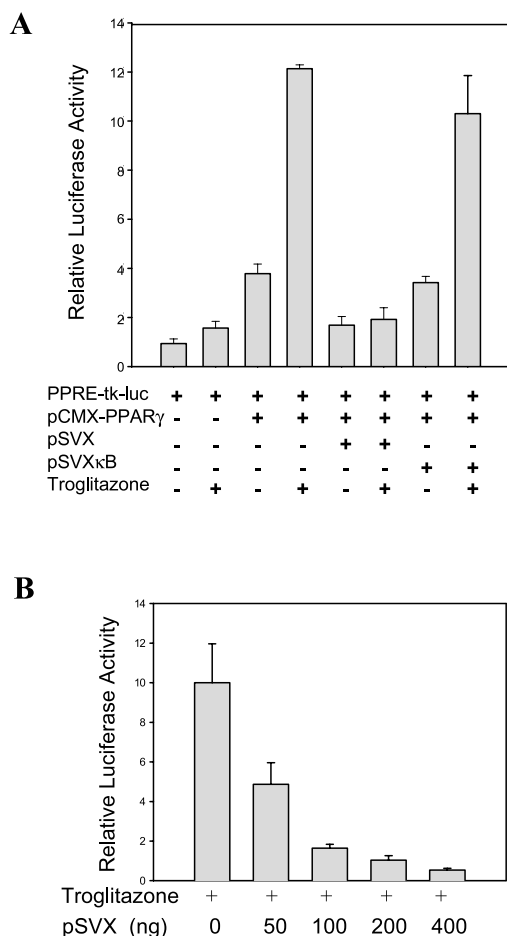


Fig. 6. Inhibition of PPAR γ transcriptional activity by HBx. Transient transfection in Chang liver cells and the luciferase reporter assay were used to examine the effect of HBx on PPAR transcriptional activity, as described in Section 2. A: Luciferase activity was found to be inhibited by the overexpression of HBx, but not by mutated HBx. Each bar was normalized versus β -galactosidase activity. Luciferase activities are represented as fold inductions relative to the basal activity of the empty vector in the absence of troglitazone. B: Dose-dependent effect of HBx on luciferase activity. Normalized values are shown as means \pm S.D. of independent experiments performed in triplicate and repeated at least three times.

in both the nuclear translocation and the DNA binding of PPAR γ .

IFA data demonstrated that PPAR γ was partially sequestered and colocalized with HBx in the cytoplasm. These results might be due to the effect of HBx on DBD and NLS at the hinge region, leading to the interruption of the nuclear localization of PPAR γ . It was observed that the amount of nuclear PPAR γ and its DNA binding activity were reduced in the NE of HBx-TG mouse liver tissues. We also found that HBx displayed an inhibitory effect on the PPRE binding of PPAR γ . The addition of rHBx into the NE of C57BL/6 reduced the DNA binding activity of PPAR γ , suggesting that nuclear PPAR γ cannot bind effectively to DNA in the presence of HBx. Overall, our results suggest that the partial cytoplasmic sequestration of PPAR γ by HBx may sufficiently reduce the concentration of nuclear PPAR γ to prevent DNA binding.

The physiological meaning of our findings needs further study, because the expression of HBx is largely confined to

liver parenchymal cells, where the expression of PPAR γ is low and where the function of PPAR γ is unclear under normal physiologic conditions. However, our study shows that HBx is responsible for the suppression of the growth inhibitory effect of troglitazone in HCC cells containing HBx. It is conceivable that the inhibitory effect of HBx on PPAR γ may be advantageous to HBx-containing hepatocytes, leading to survive the natural ligand of PPAR γ . It is also possible that HBx plays a role in biological modulation in HBV-associated diseases by interacting with and suppressing PPAR γ . According to previous reports, nuclear receptors, including PPAR γ , enhance HBV replication [41]. HBV non-responsive mutants to PPAR γ showed reduced viral replication and a lower expression level of viral antigen [42,43], however the reduced production of viral proteins may be favorable for the virus, giving it rise to evade the host immune system. These results imply that the modulation of PPAR γ by HBx can effectively regulate the life cycle of HBV and its replication. Therefore, our data suggest the possibility that HBx has an immunological or biological modulatory role in HBV-associated liver diseases, by interacting with and suppressing PPAR γ .

In the present investigation, a novel interaction between HBx and PPAR γ was studied. The results obtained from this study show that HBx suppresses the transcriptional and proapoptotic activities of PPAR γ . Recognizing the importance of PPAR γ -mediated cellular events, our results may offer a mechanism involved in the pathological effects of HBx.

Acknowledgements: We thank Drs. Ronald M. Evans, David J. Mangelsdorf, and Heonjoong Kang for providing us with PPRE $_3$ -tk-LUC and pCMX-mPPAR γ and Sankyo Co. Ltd for the troglitazone. This work was supported by a grant from the Korean National Cancer Control Program, Ministry of Health and Welfare, South Korea (02-1-2-0590).

References

- [1] Rosen, E.D. and Spiegelman, B.M. (2001) *J. Biol. Chem.* 276, 37731–37734.
- [2] DiRenzo, J., Soderstrom, M., Kurokawa, R., Ogliastro, M.H., Ricote, M., Ingrey, S., Horlein, A., Rosenfeld, M.G. and Glass, C.K. (1997) *Mol. Cell. Biol.* 17, 2166–2176.
- [3] Juge-Aubry, C., Pernin, A., Favez, T., Burger, A.G., Wahli, W., Meier, C.A. and Desvergne, B. (1997) *J. Biol. Chem.* 272, 25252–25259.
- [4] Sarraf, P., Mueller, E., Jones, D., King, F.J., DeAngelo, D.J., Partridge, J.B., Holden, S.A., Chen, L.B., Singer, S., Fletcher, C. and Spiegelman, B.M. (1998) *Nat. Med.* 4, 1046–1052.
- [5] Mueller, E., Smith, M., Sarraf, P., Kroll, T., Aiyer, A., Kaufman, D.S., Oh, W., Demetri, G., Figg, W.D., Zhou, X.P., Eng, C., Spiegelman, B.M. and Kantoff, P.W. (2000) *Proc. Natl. Acad. Sci. USA* 97, 10990–10995.
- [6] Kubota, T., Koshizuka, K., Williamson, E.A., Asou, H., Said, J.W., Holden, S., Miyoshi, I. and Koeffler, H.P. (1998) *Cancer Res.* 58, 3344–3352.
- [7] Kilgore, M.W., Tate, P.L., Rai, S., Sengoku, E. and Price, T.M. (1997) *Mol. Cell. Endocrinol.* 129, 229–235.
- [8] Tontonoz, P., Singer, S., Forman, B.M., Sarraf, P., Fletcher, J.A., Fletcher, C.D., Brun, R.P., Mueller, E., Altiock, S., Oppenheim, H., Evans, R.M. and Spiegelman, B.M. (1997) *Proc. Natl. Acad. Sci. USA* 94, 237–241.
- [9] Koga, H., Sakisaka, S., Harada, M., Takagi, T., Hanada, S., Taniguchi, E., Kawaguchi, T., Sasatomi, K., Kimura, R., Hashimoto, O., Ueno, T., Yano, H., Kojiro, M. and Sata, M. (2001) *Hepatology* 33, 1087–1097.
- [10] Rumi, M.A., Sato, H., Ishihara, S., Kawashima, K., Hamamoto, S., Kazumori, H., Okuyama, T., Fukuda, R., Nagasue, N. and Kinoshita, Y. (2001) *Br. J. Cancer* 84, 1640–1647.

- [11] Toyoda, M., Takagi, H., Horiguchi, N., Kakizaki, S., Sato, K., Takayama, H. and Mori, M. (2002) *Gut* 50, 563–567.
- [12] Cheong, J.H., Yi, M., Lin, Y. and Murakami, S. (1995) *EMBO J.* 14, 143–150.
- [13] Qadri, I., Maguire, H.F. and Siddiqui, A. (1995) *Proc. Natl. Acad. Sci. USA* 92, 1003–1007.
- [14] Haviv, I., Shamay, M., Doitsh, G. and Shaul, Y. (1998) *Mol. Cell. Biol.* 18, 1562–1569.
- [15] Perini, G., Oetjen, E. and Green, M.R. (1999) *J. Biol. Chem.* 274, 13970–13977.
- [16] Kong, H.J., Hong, S.H., Lee, M.Y., Kim, H.D., Lee, J.W. and Cheong, J. (2000) *FEBS Lett.* 483, 114–118.
- [17] Truant, R., Antunovic, J., Greenblatt, J., Prives, C. and Cromlish, J.A. (1995) *J. Virol.* 69, 1851–1859.
- [18] Elmore, L.W., Hancock, A.R., Chang, S.F., Wang, X.W., Chang, S., Callahan, C.P., Geller, D.A., Will, H. and Harris, C.C. (1997) *Proc. Natl. Acad. Sci. USA* 94, 14707–14712.
- [19] Yun, C., Lee, J.H., Park, H., Jin, Y.M., Park, S., Park, K. and Cho, H. (2000) *Oncogene* 19, 5163–5172.
- [20] Wang, X.W., Forrester, K., Yeh, H., Feitelson, M.A., Gu, J.R. and Harris, C.C. (1994) *Proc. Natl. Acad. Sci. USA* 91, 2230–2234.
- [21] Seifer, M. and Gerlich, W.H. (1992) *Arch. Virol.* 126, 119–128.
- [22] Kim, C.M., Koike, K., Saito, I., Miyamura, T. and Jay, G. (1991) *Nature* 351, 317–320.
- [23] Koike, K., Moriya, K., Iino, S., Yotsuyanagi, H., Endo, Y., Miyamura, T. and Kurokawa, K. (1994) *Hepatology* 19, 810–819.
- [24] Yu, D.Y., Moon, H.B., Son, J.K., Jeong, S., Yu, S.L., Yoon, H., Han, Y.M., Lee, C.S., Park, J.S., Lee, C.H., Hyun, B.H., Murakami, S. and Lee, K.K. (1999) *J. Hepatol.* 31, 123–132.
- [25] Diao, J., Garces, R. and Richardson, C.D. (2001) *Cytokine Growth Factor Rev.* 12, 189–205.
- [26] Sells, M.A., Chen, M.L. and Acs, G. (1987) *Proc. Natl. Acad. Sci. USA* 84, 1005–1009.
- [27] Klierer, S.A., Forman, B.M., Blumberg, B., Ong, E.S., Borgmeyer, U., Mangelsdorf, D.J., Umesono, K. and Evans, R.M. (1994) *Proc. Natl. Acad. Sci. USA* 91, 7355–7359.
- [28] Spandau, D.F. and Lee, C.H. (1988) *J. Virol.* 62, 427–434.
- [29] He, T.C., Zhou, S., da Costa, L.T., Yu, J., Kinzler, K.W. and Vogelstein, B. (1998) *Proc. Natl. Acad. Sci. USA* 95, 2509–2514.
- [30] Kim, J., Chwae, Y.J., Kim, M.Y., Choi, I.H., Park, J.H. and Kim, S.J. (1997) *J. Immunol.* 159, 3875–3882.
- [31] Lee, M.O., Choi, Y.H., Shin, E.C., Kang, H.J., Kim, Y.M., Jeong, S.Y., Seong, J.K., Yu, D.Y., Cho, H., Park, J.H. and Kim, S.J. (2002) *J. Hepatol.* 37, 380–386.
- [32] Kim, H.I., Kim, J.W., Kim, S.H., Cha, J.Y., Kim, K.S. and Ahn, Y.H. (2000) *Diabetes* 49, 1517–1524.
- [33] Jiang, S., Song, M.J., Shin, E.C., Lee, M.O., Kim, S.J. and Park, J.H. (1999) *Hepatology* 29, 101–110.
- [34] Hsu, M.H., Palmer, C.N., Song, W., Griffin, K.J. and Johnson, E.F. (1998) *J. Biol. Chem.* 273, 27988–27997.
- [35] Altucci, L. and Gronemeyer, H. (2001) *Trends Endocrinol. Metab.* 12, 460–468.
- [36] Fajas, L., Debril, M.B. and Auwerx, J. (2001) *J. Mol. Endocrinol.* 27, 1–9.
- [37] Makino, Y., Yoshikawa, N., Okamoto, K., Hirota, K., Yodoi, J., Makino, I. and Tanaka, H. (1999) *J. Biol. Chem.* 274, 3182–3188.
- [38] Moilanen, A.M., Poukka, H., Karvonen, U., Hakli, M., Janne, O.A. and Palvimo, J.J. (1998) *Mol. Cell. Biol.* 18, 5128–5139.
- [39] Tsutsumi, T., Suzuki, T., Shimoike, T., Suzuki, R., Moriya, K., Shintani, Y., Fujie, H., Matsuura, Y., Koike, K. and Miyamura, T. (2002) *Hepatology* 35, 937–946.
- [40] Gampe Jr., R.T., Montana, V.G., Lambert, M.H., Miller, A.B., Bledsoe, R.K., Milburn, M.V., Klierer, S.A., Willson, T.M. and Xu, H.E. (2000) *Mol. Cell* 5, 545–555.
- [41] Yu, X. and Mertz, J.E. (1997) *J. Virol.* 71, 9366–9374.
- [42] Yu, X. and Mertz, J.E. (2001) *J. Virol.* 75, 11354–11364.
- [43] Raney, A.K., Kline, E.F., Tang, H. and McLachlan, A. (2001) *Virology* 289, 239–251.